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COP1 interacts with the ELF4-ELF3-LUX complex for the regulation of hypocotyl elongation in *Arabidopsis thaliana*

BY

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ABSTRACT

Daily rhythms are regulated by endogenous circadian clock in plants. A circadian oscillator keeps circadian time regarding the appropriate time of day (24-h) and is entrained by environmental cues such as daily and seasonal changes.

The most circadian clock is showing rhythmical pattern which can regulate hypocotyl elongation in *Arabidopsis thaliana*. In previous study, ELF3-ELF4-LUX complex (EC complex) functions as a circadian clock and represses the expression of the bHLF factors *PHYTOCHROME INTERACTING FACTOR* (*PIF*). *ELF3* (*EARLY FLOWERING 3*), *ELF4* (*EARLY FLOWERING 4*) and *LUX* (*LUX ARRHYTHMMO*; *PHYTOCLOCK 1*) (called the evening complex) form the protein complex in the early evening. It is required for the proper expression of the transcription factors. *PIF4* and *PIF5* genes are belong to these types of transcription factors. EC complex inhibits the *PIF4* and *PIF5*, leading to promoting photomorphogenesis; yet, the mechanism by which between COP1 and EC complex is still unknown. Here, we elucidate the regulation of hypocotyl length by interaction between EC complex and *PIFs* through COP1. To figure out EC complex can regulate hypocotyl elongation through COP1, known as a repressor of photomorphogenesis, we investigated phenotypes of hypocotyl length. Based on hypocotyl phenotypes under LD, SD, 12L/12D and DD conditions, the phenotypes of *cop1-4* mutant are shorter than these of wild type (Col-

0). Also, the phenotypes of *elf3-1*, *elf4-101* and *pcl1-1* (evening complex) mutants are longer than these of wild type. In other words, a loss-function of *COP1* gene leads to short hypocotyl as repressors of photomorphogenesis. To identify genetic analysis between *COP1* and *EC* complex, we constructed various mutant lines containing double (*cop1-4elf3-1*, *cop1-4elf4-101*, *cop1-4pcl1-1*) and triple (*cop1-4elf3-1pcl1-1*) mutants. When double and triple mutants were analyzed, we found that phenotypes of *cop1-4* background mutant including *cop1-4elf3-1*, *cop1-4elf4-101*, *cop1-4pcl1-1*, *cop1-4elf3-1elf4-101* were shorter than those of wild type, indicating that *COP1* acts downstream of *ELF3*, *ELF4* and *LUX*. To investigate relationship between *EC* complex and *COP1,* we examined *COP1* mRNA level in *elf3-1*, *elf4-101*, and *pcl1-1* mutants under 12L/12D (or 8h light/16h dark) condition. There was no difference expression of *COP1* mRNA in evening complex mutants compared with Col-0. To investigate relationships between *COP1* and *PIF,* we showed that the expressions of *PIFs* mRNA were regulated by *COP1* under 12L/12D conditions. For this reason, *PIFs* direct-gene expressions were also induced, suggesting that *COP1* functions as activator of *PIFs* genes. These data showed that *COP1* with EC complex regulated *PIFs* mRNA expressions. In this study, the expression of *COP1* mRNA in evening complex mutants is no difference compared with Col-0, however genetic interaction following *cop1-4* mutant phenotype was clearly showed. Therefore, we suggest that between COP1 and EC complex is associate with translational regulation. To demonstrate translational relationship between EC complex and COP1, we examined yeast-two hybrid assays. We found that COP1 physically interacted with ELF3, ELF4 and LUX. Thus, we propose a model that COP1 is translationally supperesed by EC complex. Also COP1 can transcriptionally activates *PIFs* mRNA levels. Overall, these results provide a molecular mechanism for the hypocotyl elongation by COP1. And

EC complex through *PIFs* pathway is mediated photomorphogenesis.

Keywords: hypocotyl elongation, circadian clock, Evening complex, *PHYTOCHROME INTERACTING FACTOR, EARLY FLOWERING 3, EARLY FLOWERING 4, CONSTITUTIVELY PHOTOMORPHOGENIC 1, LUX ARRHYTHMMO,*

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ABBREVIATION

EC complex	Evening Complex (ELF4-ELF3-LUX)
12L/12D	12 h light / 12 h dark condition
8L/16D	8 h light / 16 h dark condition (Short-day / SD)
16L/8D	16 h light / 8 h dark conditions (Long-day / LD)
DD	Continuous dark condition
COP1	Constitutively Photomorphogenic 1
ELF3	Early Flowering 3
ELF4	Early Flowering 4
LUX	LUX ARRHYTHMMO; PHYTOCLOCK 1
PIF	Phytochrome Interacting Factor
ZT	Zeitgeber time
MS	Murashige & Skoog

INTRODUCTION

Many scientists use *Arabidopsis thaliana* in their studies, which is known as an important model plant. It is used to study identifying genes and determining its functions. In general, plants can be classified as long-day (LD), short-day (SD) and day-neutral plants according to light length. Light is an important environmental factor for plants. It provides energy source for plants that photosynthesis is used to adapt their growth and development with environmental changes such as the intensity of daylight and temperature [1],[2]. Many plants have the circadian rhythms according to light, which are an endogenous molecular oscillator with the approximately 24-h fluctuations. This oscillator, known as the circadian clock, generates circadian rhythms that are affected by environmental factors such as light, and temperature [3, 4]. By the circadian clock, regulated rhythmical genes including EC complex are associated with hypocotyl elongation, cotyledon movement, and stomatal opening in photomorphogenesis.

In *Arabidopsis*, light length is an important regulator of hypocotyl elongation. In light conditions, the seedlings exhibit open and expanded cotyledons, a short hypocotyl, chloroplast development, and a high expression level for light-inducible genes [5-7]. However, in dark conditions, the seedlings show closed and undeveloped cotyledons, a long hypocotyl, low or undetectable

level for light-inducible genes [8-11].

The evening proteins ELF3 (EARLY FLOWERING3), ELF4 (EARLY FLOWERING), the transcription factor *LUX* (*LUX ARRHYTHMMO*; *PHYTOCLOCK 1*) form the Evening Complex (EC complex) [12]. ELF3 has a crucial bridge role which is required to interaction between LUX and ELF4. ELF3 and ELF4 are localized in nucleus and regulate circadian rhythms [13], [14]. *LUX* is a single MYB domain-containing SHAQYF-type GARP transcription factor [15]. EC complex affects the circadian oscillator, regulating hypocotyl elongation under the light length. This complex regulates hypocotyl elongation through *PIF4* and *PIF5* genes [16, 17]. In the early night, EC complex represses *PIF4* and *PIF5* mRNA levels which are known as basic helix-loop-helix (bHLH) transcription factors, leading to short hypocotyl phenotype [18].

Genetic screens for various mutant lines that show photomorphogenic development in darkness resulted in the identification of CONSTITUTIVE PHOTOMORPHOGENIC/DE-ETIOLATED/FUSCA (COP/DET/FUS) gene family [19-23]. COP/DET/FUS genes act as negative regulators of photomorphogenesis in darkness [20]. The *cop1-4* mutant has etiolated phenotype, including short hypocotyl, open and enlarged cotyledons, no apical hook, and accumulate high levels of anthocyanin under dark conditions [24]. COP1 was first found as a repressor of seedlings photomorphogenesis in darkness [25]. COP1 acts as a RING-type E3 ubiquitin ligase that mediates

ubiquitination and targeted degradation [26-28]. Protein size of COP1 is a 76-kD that contains three domains including single RING-finger, coiled-coil, and WD40 domain [29-31].

In plant morphogenesis, the molecular mechanism controlling hypocotyl elongation is poorly understand [32]. Here, we demonstrate that EC complex represses *PIFs* through COP1 for the regulation hypocotyl elongation in the photomotphogenesis. Thus, we examined relationship between EC complex and COP1 to photomorphogenesis by genetic and biochemical approaches, including phenotypes of genetic interaction, yeast two hybrid assays, and transcriptional regulation assay. We found that two of EC complex physically interact with COP1. Up to now, ELF3 gene has been studied in photomorphogenesis. However, how ELF4 and LUX genes have function in *arabidopsis thaliana* is not clear. Therefore, we suggest that a new function between EC complex and *PIFs* through COP1 in the photomorphogenic development in this study.

MATERIALS AND METHODS

Seedling growth and hypocotyl measurements

All the *Arabidopsis thaliana* lines have Columbia (Col-0) genetic background in this study. To experiment involving plants grown under sterile conditions, seeds were surface-sterilized and plated on murashige and skoog salts (MS) medium (Duchefa). To create double and triple mutants, F_1 heterozygotes were obtained by crossing *cop1-4* as the female plant with other evening complex mutants including *elf3-1*, *elf4-101*, and *pcl1-1* (*lux*) mutants as pollen donors. To select correct transformants, the plants showing morphological phenotype of *cop1-4* were first isolated among F_3 plants, in which flowering-time mutations were finally confirmed by PCR-based genotyping. In all growth chambers, seedlings were also grown under continuous white light at 100μmol$m^{-2}s^{-1}$. Dark-grown seedlings were kept for 3 d in foil-covered plates. Surface-sterilized seeds were plated onto 1 X Murashige and Shoog (MS) basal salt medium with 1.5% agar and 2% sucrose. After stratification in the dark at 4℃ for 4 days, plates were transferred to an incubator that was set to the indicated light conditions and a constant temperature of 22℃. Seedlings were grown in 12L/12D (12h light/12h dark), LD (16h light/8h dark), SD (8h

light/16h dark), and DD conditions at 22℃. For hypocotyl measurements, seedlings were arranged horizontally on a plate, photographed using a digital camera. At least 20 seedlings for each line were measured to calculate the mean and strandard error.

RNA extraction and quantitative real-time PCR analysis

Seedlings were grown on Whatman filter paper atop MS plates under each conditions and harvested every 4h at 22℃. Total RNA was isolated using an Total RNA Extraction mini kit (MGmed, South Korea), including the on-column DNase digestion step according to the manufacturer's protocols. First strand cDNA was synthesized from 2μg total RNA using oligo $(dT)_{15}$ primer and M-MLV reverse transcriptase (Promega). Transcript levels of photomorphogenesis-related genes were detected by qRT-PCR using gene-specific primers and *ACTIN 2* (*ACT2*) as internal control (Table 1). Total 20 μl of mixture included 2 μl of 0.5 μM primer, 2 μl of cDNA mixture and 10 μl of 2X QuantiTect LightCycler 480 SYBR Green I Master Mix (Roche). PCR was performed with Light Cycler 2.0 instrument (Roche Diagnostics) using the following program: 95°C for 2 min, 45 cycles of 95°C for 10 sec, 59°C for 10 sec and 72°C for 10 sec. Each PCR was repeated at least three times using

biologically independent samples.

Yeast two-hybrid assays

The full-length cDNAs of *COP1*, *ELF3*, *ELF4*, and *LUX* were amplified from wild-type (Col-0) total RNA using RT-PCR. The full length of COP1 (aa;1-2028) was used as bait in pGBK vector. For preys, it was used the full length of ELF3 (aa;1-2088), ELF4 (aa;1-336), and LUX(aa;1-975). Yeast co-transformation and LacZ activity assays were performed according to the manufacturer's protocol. The PCR products were cloned into pGBKT7 and pGADT7 vectors (MATCHMAKER GAL4 TWO-hybrid system 3, Clontech) to get the bait and prey clones. For the interaction study, plasmids containing fusion proteins were transformed into Saccharomyces cerevisiae AH109 and grown on media lacking adenine, leucine, histidine, and tryptophan SD based agar plates at 30℃ for 4days. For β-galactosidase activity assay, three independent colonies were picked up from -4 (Ade/Leu/His/Trp) agar plates and inoculated into -2 (Leu/Trp) liquid media, and were shaken at 30℃ until density reached OD600 of approximately 1.5nm.

Table 1. Information of primers used for qRT-PCR in this study

Primer name	Forward (5' - 3')	Reverse (5' - 3')
PIF1-qRT	GATGAAATGACTTCTTGGCTTCAT	AAGATCTGAGCAGAAATCATCGT
PIF3-qRT	GATCATGTCAATGGCGTCTG	CATACCCGGTGGGAACATAA
PIF4-qRT	GCGAGATGGACAAGTGGTTC	TTCTGGGTTTGGGTTTGTTC
PIF5-qRT	GATTTTTGTTACGTGTGAAGCAAT	CTTGTTCCATGTCAGATCAGATTTA
COP1-qRT	TTCAGCCAACATTGTATCAAGC	AAACACCAGCAGTGGCAAA
ARF18-qRT	AGAGGCAACAAGACAGTTGCTGAGG	ACTTCAGGGAGGTCCTTGCAAGGTT
ATHB2-qRT	CGACGACAATAGCTCGATCGC	GTGCATGGTCACGGAGCCTT
EDF3-qRT	CAGGCCAGGAATAAGCTGGAT	TTAGGATGGTTTGGTGTCATTGC
PIL2-qRT	ATCGTTCTGCAGGCCGAGA	TCACCAATGCTTCTGGGCTAT
SDR-qRT	GGACCACCTCGTCACCTACAA	CGCCGTTGGCCATGAG
ST2A-qRT	CGTGCAATGGTTTAGACTAAAG	AACAGCATCAGCATCAACAA
XTR7-qRT	AGGTGCTACGAGAAGCAAATCC	GGGCCTCATCTCGGCATAG
IAA19-qRT	TCAGCAACAGCATATCTTTCTCATCA	TCTGGAATTTGGCATATCTATCACC
SNRK2.5-qRT	GGATGCAAGGAAATCATGGA	AATCCCATCGGAAATCTTGG
ACT2-qRT	TGGGATGAACCAGAAGGATG	AAGAATACCTCTCTTGGATTGTGC

RESULTS

CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) Is Epistatic to *EVENING COMPLEX* in the Photomorphogenesis Pathway of Hypocotyl elongation

In general, *Arabidopsis thaliana* has a hypocotyl length in photomorphogenesis much longer in dark than in light. In previous study, the *cop1* mutant has a short hypocotyl length [33, 34], while the *elf3*, *elf4*, *pcl1* (*lux*), mutants show long hypocotyl phenotypes [12, 15, 35]. The *cop1-4* has been isolated as one of the *cop1* mutant alleles, which has a mutation in the exon of *COP1*, resulting in a weak allele. *cop1-4* mutant displays photomorphogenic development in darkness, with phenotype such as short hypocotyls and opened cotyledons [22, 25]. Because *EC* complex represses *PIF4* and *PIF5* mRNA levels during the early evening in photomorphogenic pathway in the 12h light/12h dark (12L/12D) condition [12, 17], we examined hypocotyl length for wild-type (Columbia-0 [Col-0]) and mutant seedlings grown under 12L/12D condition including other conditions (Long day, Short day, Continuous dark conditions). We first found that under 12L/12D, *cop1-4* mutant showed a shorter hypocotyl, with 1.82 ± 0.41mm, about 0.77mm than wild type (WT, 2.59 ± 0.46mm). By contrast, *elf3-1*, *elf4-101,* and *pcl1-*

1 mutants showed longer hypocotyls, with 7.29 ± 0.65mm, 3.36 ± 0.53mm, 6.07 ± 0.84mm respectively, than wild type (Figures 1A to 1B). Second, we detected that under 8h light/16h dark (SD) condition, *cop1-4* mutant showed shorter hypocotyl phenotype, with 0.53 $\pm$ 0.06mm, about 2.5mm than wild type (WT, 3.03 $\pm$ 0.47mm). By contrast, *elf3-1*, *elf4-101,* and *pcl1-1* mutants displayed longer hypocotyl phenotypes, with 8.13 ± 1.00mm, 4.11 ± 0.56mm, 5.85 ± 2.49mm respectively, than wild type (Figures 1C to 1D). Third, under continuous dark (DD) condition, *cop1-4* mutant also showed shorter hypocotyl phenotype, with 3.75 $\pm$ 0.63mm, about 5.32mm than wild type (WT, 9.07 $\pm$ 1.15mm). By contrast, *elf3-1*, *elf4-101* and *pcl1-1* mutants showed longer hypocotyls, with 9.51 ± 1.71mm, 9.49 ± 0.98mm, 8.83 ± 1.30mm respectively, than wild type (Figure 2A). Fourth, we detected that under 16h light/8h dark (LD) condition, *cop1-4* mutant showed shorter hypocotyl length, with 0.74 $\pm$ 0.15mm, about 0.33mm than wild type (WT, 1.07 $\pm$ 0.30mm). By contrast, *elf3-1*, *elf4-101* and *pcl1-1* mutants displayed longer hypocotyl lengths, with 3.59 ± 0.33mm, 1.35 ± 0.25mm, 1.95 ± 0.25mm respectively, than wild type (Figure 2B). These results indicated that *COP1* acts as a photomorphogenetic repressor under every conditions (12L/12D, SD, LD ,DD) and plays an important role in regulating hypocotyl elongation in *Arabidopsis thaliana*.

As observed previously, the *elf3-1*, *elf4-101* and *pcl1-1* mutants displayed longer hypocotyl lengths than the wild type [Col-0], while the *cop1-4* displayed

shorter hypocotyl length than wild type (Figures 1 to 2). To test whether *COP1* genetically interacts with *EC* complex, we generated various combinations of *cop1-4* background mutant such as *cop1-4elf3-1*, *cop1-4elf4-101*, *cop1-4pcl1-1* double and *cop1-4elf3-1elf4-101* triple mutants. Unfortunately, we didn't have *cop1-4elf3-1elf4-101pcl1-1* quadruple mutant. However, because we had *cop1-4elf3-1elf4-101* triple mutant, we could perform that the triple mutant also revealed the *cop1-4* mutant phenotype (Figures 1 to 2). We detected that under 12L/12D condition, *cop1-4elf3-1* mutant showed shorter hypocotyl phenotype, with 2.08±0.4mm, about 0.51mm than wild type (WT, 2.59 ± 0.46mm). *cop1-4elf4-101* mutant showed shorter hypocotyl phenotype, with 1.27±0.36mm, about 1.32mm than wild type. *cop1-4pcl1-1* mutant showed shorter hypocotyl phenotype, with 2.11 ± 0.21mm, about 0.48mm than wild type. *cop1-4elf3-1elf4-101* mutant showed shorter hypocotyl length, with 2.27±0.36mm, about 0.32mm than wild type (Figures 1A to 1B). Under SD condition, *cop1-4elf3-1, cop1-4elf4-101 and cop1-4pcl1-1* mutants also showed shorter hypocotyl phenotypes, with $1.06 \pm 0.18\mathrm{mm}$, $0.87 \pm 0.12\mathrm{mm}$, $0.72 \pm 0.16\mathrm{mm}$ respectively, than wild type (WT, 3.03 ± 0.47mm). *cop1-4elf3-1elf4-101* mutant showed short hypocotyl phenotype, with 1.08±0.22mm, compared with WT (Figure 1C to 1D). Under DD condition, *cop1-4elf3-1, cop1-4elf4-101 and cop1-4pcl1-1* mutants also showed shorter hypocotyl phenotypes, with $4.38 \pm 0.86\mathrm{mm}$, $4.83 \pm 0.59\mathrm{mm}$, $4.65 \pm 0.96\mathrm{mm}$, respectively, than wild type (WT, 9.07 ± 1.15mm). *cop1-4elf3-*

1elf4-101 mutant showed short hypocotyl phenotype, with 4.94±0.85mm, compared with WT (Figure 2A). Under LD condition, *cop1-4elf3-1, cop1-4elf4-101 and cop1-4pcl1-1* mutants also showed shorter hypocotyl phenotypes, with $1.08 \pm 0.22\mathrm{mm}$, $0.63 \pm 0.13\mathrm{mm}$, $0.69 \pm 0.16\mathrm{mm}$ respectively, than wild type (WT, 1.07 ± 0.30mm). *cop1-4elf3-1elf4-101* mutant showed short hypocotyl phenotype, with 0.87±0.21mm, compared with WT (Figure 2B).

In this results, double and triple mutants displayed *cop1-4* mutant phenotype (Figures 1 to 2). So, we can imagine that quadruple mutant is the same phenotype as the triple mutant. Taken together, these phenotypes data showed us that *COP1* acts genetically downstream of *ELF3/ELF4/LUX* in hypocotyl elongation of photomorphogenesis.

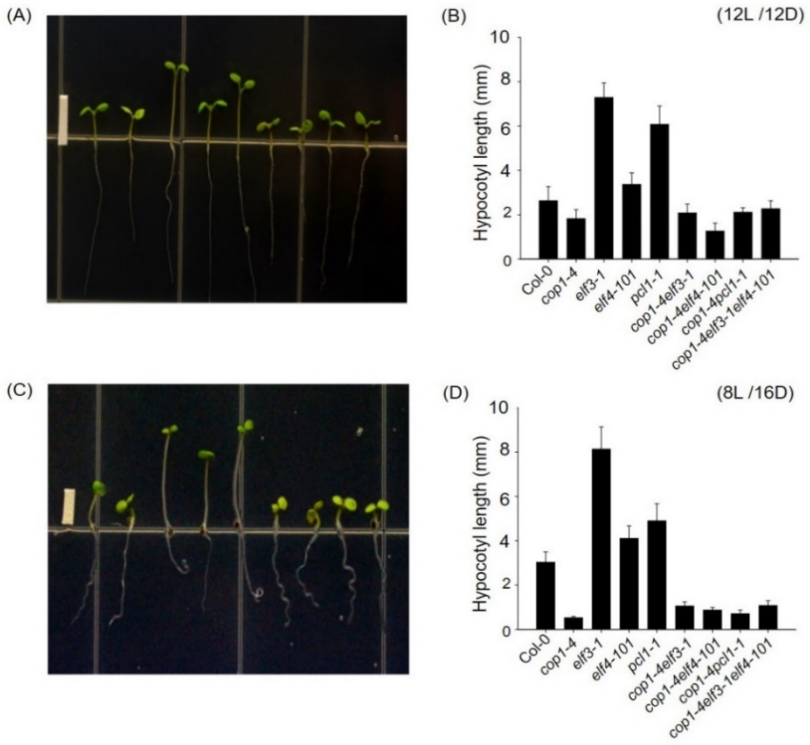

Figure 1. *EVENING COMPLEX* genetically acts upstream of *COP1* in regulating hypocotyl growth.

(A-B) Visible phenotypes of the wild type (Col-0), various single (*cop1-4*, *elf3-1*, *elf4-101*, *pcl1-1)*, double (*cop1-4elf3-1*, *cop1-4elf4-101*, *cop1-4pcl1-1*), triple (*cop1-4elf3-1elf4-101*) mutants. Seeds of various genotypes were grown on MS medium with 2% sucrose for 5d-old-seedlings in the 12h light/ 12h dark condition (A-B) and for 5d-old-seedlings in the 8h light/ 16h dark condition (C-D). Plants were grown at 22℃ growth chamber under white fluorescent light (100 $\mu molm^{-2}s^{-1}$). Photographs show the hypocotyl length. Means and standard deviations were obtained from at least 20 seedlings. Scale bars = 3 mm.

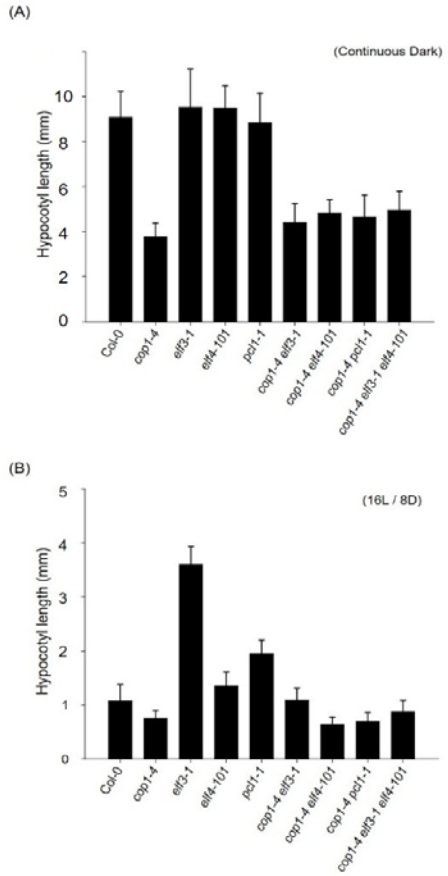

Figure 2. Genetic analysis of hypocotyl lengths in Col-0 and *cop1-4* background mutants.

(A) Visible phenotypes of the wild type (Col-0) and various single, double, triple mutant lines. Seeds of various genotypes were grown on MS medium with 2% sucrose for 3d-old-seedlings in the continuous dark condition and (B) 8d-old-seedlings in the 16h light/ 8h dark condition. Plants were grown at 22℃ growth chamber under white fluorescent light (100 $\mu molm^{-2}s^{-1}$). Photographs show the hypocotyl length. Means and standard deviations were obtained from at least 20 seedlings. Scale bars = 3 mm.

COP1 activates the transcription of *PIFs* gene expression.

In the dark, photomorphogenesis for hypocotyl elongation is controlled by at least two mechanisms, which are *HY5* and *PIF* pathway [36, 37]. In plant development, COP1 E3 ubiquitin ligase degrades several photomorphogenesis boosting transcription factors including *HY5*, *HFR1*, *LAF1* [28, 38], but not others leading etiolated growth, such as the *PIF1*, *PIF3* and *PIF4* [39], well known as phytochrome interacting bHLH transcription factors. In previous study, *PIFs* genes are degraded and phosphorylated by light inducting phytochrome such as *PHYA* and *PHYB* [40, 41].

Many bHLH transcription factors are important for the morphogenesis such as hypocotyl elongation. In the dark, COP1 supports the stabilization of PIF3 and other PIF factors [42] . Moreover, previous study prove that the basic helix-loop-helix transcription factors *PIF4* and *PIF5* are vital for determining the hypocotyl elongation rate in seedlings [12, 43, 44]. And EC complex negatively regulates *PIF4* and *PIF5* transcriptional levels [12, 17]. To investigate relationship between *COP1* and *PIFs*, we performed qRT-PCR using samples harvested every 4hours to analyze up to four genes of *PIF1*, *PIF3*, *PIF4*, and *PIF5*. To perform this experiment, we raised two kinds of seedlings (; Col-0 and *cop1-4*) in 12L/12D for 12d-old-seedlings. The abundance of mRNA was quantified by qRT-PCR and expressed relative to the abudance of *ACTIN2* transcripts. Seedlings were grown at 22℃ growth

chamber under white fluorescent light(100μ mol $m^{-2}s^{-1}$). Means and standard deviations value of three repliciates are shown.

In 12L/12D condition, we measured the transcript levels of *PIFs* mRNA every 4hours in a 24h cycle starting at the onset of light in these genotypes. *PIFs* genes containing *PIF1*, *PIF3*, *PIF4*, and *PIF5* are mostly downregulated in *cop1-4* compared with wild-type, in specially ZT12 point time where EC complex forms in 12L/12D condition (Figures 3A-3D). We found that the transcript levels of *PIF1* and *PIF4* at ZT8 (zeitgeber time; 8 h) were almost the same in wild-type [Col-0] and *cop1-4* mutant. The Col-0 showed higher expressions of *PIF1* and *PIF4* mRNA levels after ZT0 in the light, while the *PIF1* and *PIF4* mRNA levels in Col-0 observed lower expressions after ZT12 in the dark (Figures 3A and 3C). Likewise, *PIF1* and *PIF4* genes were also showed to increased and decreased pattern in *cop1-4* mutant (Figures 3A and 3C). The transcript level of *PIF3* increased at ZTZ8 and significantly decreased in *cop1-4* mutant compared with WT (Figure 3B). *PIF5* mRNA level in *cop1-4* increased at ZT4 and decreased after ZT4 (Figure 3D). Over all, the gap between WT and *cop1-4* significantly decreased in the dark (Figures 3A and 3B). The gap between WT and *cop1-4* significantly increased from ZT0 to ZT8 while the gap slightly decreased from ZT12 to ZT20 (Figures 3C and 3D). The transcript levels of *PIFs* generally decreased in *cop1-4* mutant compared with WT (Figure 3). In previous study, EC complex forms particularly during the early evening (at ZT12). According to this study, we

focus on ZT12 time point where the evening complex is composed. All *PIFs* genes were downregulated after ZT12 time point. Taken together, these results reveal the conclusion in which COP1 regulates *PIFs* mRNA levels.

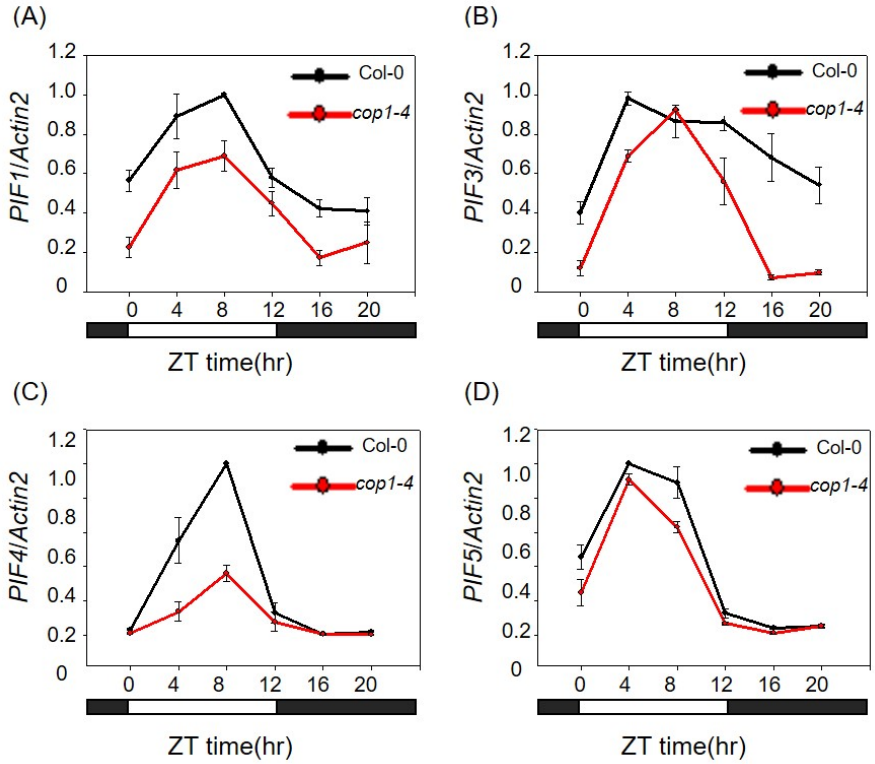

Figure 3. COP1 regulates the transcription levels of *PIFs* genes.

(A-D) The expression of *PIF1* (A), *PIF3* (B), *PIF4* (C), and *PIF5* (D) genes was analyzed in Col-0 and *cop1-4* mutant by real-time PCR using 12d-old seedlings. Plants were grown at 22℃ growth chamber under 12L/12D (12h light;12h dark) condition, and plant tissues were harvested every 4hours. *ACTIN2* expression was used for normalization. Means and standard deviations were obtained from three biological replicates. These experiments were repeated twice with the same results.

COP1 activates the transcription of *PIF* direct-target genes.

A protein complex (called the EVENING COMPLEX)-comprised of the proteins encoded by ELF3/ELF4/LUX [12, 17]. Protein accumulation peak time of the EC complex was start at ZT12 in 12 h light and 12 h dark conditions (12L/12D). Now that we figure *COP1* activates *PIFs* transcriptional levels (Figures 3A to 3D), we examine whether *COP1* might also regulate the expression of *PIF* direct-target genes. If *EC* complex represses *COP1* activity, we suggest that its activity should be reflected in reduced *PIFs* mRNA levels. To test this possibility, we performed qRT-PCR to analyze up to nine direct targets of *PIFs* at ZT16 point time [45] and among those targets especially *PIL2* and *XTR7* are displayed to regulate hypocotyl elongation [42, 46]. *PIF3* regulates to *ARF18*, *SNRK2.5* mRNA levels and *PIF4* contributes to *ATHB2*, *XTR7* mRNA levels. These *PIF* bHLH factors provide a transcriptional level that regulates seedling morphogenesis of hypocotyl elongation in *Arabidopsis*.

ARF18 transcript was 5 times detected in Col-0 (with 0.4 ± 0.12) more than in *cop1-4* (with 0.08 ± 0.01). The expression level of *ATHB2* in Col-0 (with0.35 ± 0.05) was about 3 times higher than that of *ATHB2* in *cop1-4* (with 0.11 ± 0.01) single mutant. The expression level of *EDF3* in *cop1-4* (with 0.16 ± 0.06) was about 1.6 times lower than that of *EDF3* in the wild-type (with 0.26 ± 0.08). The expression of *PIL2* was 1.8 times increased in Col-0 (with 0.34 ± 0.06) more than *cop1-4* (with 0.19 ± 0.02). Also, the expression

of *IAA19* was increased 1.8 times in Col-0 (with 0.25 ± 0.09) more than *cop1-4* (with 0.14 ± 0.01). *SDR* mRNA level decreased about 2 times in *cop1-4* (with 0.47 ± 0.10) mutant compared with wild-type (with 0.93 ± 0.10). *ST2A* mRNA level was about 3.8 times upregulated in Col-0 (with 0.46 ± 0.31) compared with *cop1-4* (with 0.12 ± 0.01) mutant. The expression level of *XTR7* in Col-0 (with 0.85 ± 0.19) was significantly about 4 times higher than that of *XTR7* in *cop1-4* (with 0.2 ± 0.05) single mutant. The expression of *SNRK2.5* was increased about 2 times in Col-0 (with 0.62 ± 0.08) compared with *cop1-4* (with 0.3 ± 0.04). All these genes were strongly downregulated on ZT16 in *cop1-4* mutant compared to Col-0 (Figure 3). Because COP1 activates *PIFs* mRNA levels, *PIF*-direct genes also down-regulate expression in *cop1-4* mutant (Figure 4). These results indicated that hypocotyl elongation was controlled by *PIF* in *cop1-4* mutant. Thus, we speculated that hypocotyl length of *cop1-4* mutant was extremely shorted in every conditions (Figures 1 and 2). Taken together, these results indicate that COP1 regulates hypocotyl elongation controlled by *PIF* in the photomorphogenesis.

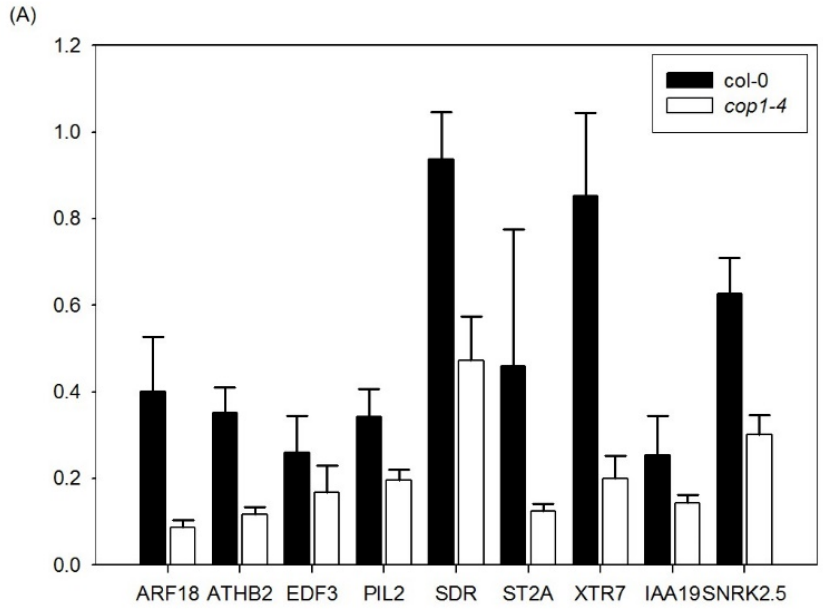

Figure 4. *COP1* regulates the transcription levels of *PIF* direct-target genes.

(A) The expressions of *ARF18*, *ATHB2*, *EDF3*, *PIL2*, *SDR*, *ST2A*, *XTR7*, *IAA19* and *SNRK2.5* genes were analyzed in Col-0 (Black bars) and *cop1-4* (White bars) mutants by real-time PCR using 12d-old seedlings. Plants were grown at 22℃ growth chamber under 12L/12D (12h light;12h dark) conditions, and plant tissues were harvested at ZT16. *ACTIN2* expression was used for normalization. Means and standard deviations were obtained from biological replicates.

ELF3 represses *PIF4* and *PIF5* through COP1 in Photomorphogenesis.

In previous study, EVENING COMPLEX (ELF3/ELF4/LUX) participated in the modulation of *PIF4* and *PIF5* mRNA levels [12, 17]. So we were focused to study *PIF4* and *PIF5* genes in *cop1-4*, *elf3-1*, *cop1-4elf3-1* and wild type. Prior to this experiment, we raised four kinds of seedlings (; *cop1-4*, *elf3-1*, *cop1-4elf3-1* and Columbia-0) in 12L/12D (12h light/12h dark) and SD (8h light/16h dark). At first, we checked the expression pattern of *PIF4* and *PIF5* in various mutant lines. To compare the expression levels of *PIF4* and *PIF5* in wild type with in *cop1-4*, *elf3-1*, *cop1-4elf3-1* mutants, we perfomed Quatitative Real-Time PCR (qRT-PCR) using samples harvested at intervals of four hours. Total RNA samples were collected from 12-d-old or 5-d-old seedlings contained in 12L/12D or SD condition of 24hr, respectively. The abundance of mRNA was quantified by qRT-PCR and expressed relative to the abudance of *ACTIN2* transcripts (Figures 5A-D). Seedlings were grown at 22℃ under white fluorescent light(100μ mol $m^{-2}s^{-1}$). Means and standard deviations value of three repliciates are shown.

We focused on *PIF4* and *PIF5* mRNA levels of mutant lines after ZT12. In 12L/12D condition, the transcript level of *PIF4* was highest at ZT8 (zeitgeber time; 8h) and decreased after ZT12 (zeitgeber time; 12h) in Col-0 (Figure 5A). The *cop1-4* mutant seedling slightly showed down-regulated *PIF4* mRNA

expression compared with Col-0 (Figure 5A). However, *elf3-1* mutant seemed to up-regulated *PIF4* mRNA expression compared with Col-0 in the dark (Figure 5A). The *cop1-4elf3-1* double mutant seedlings observed up-regulated *PIF4* mRNA expression compared with *cop1-4* and down-regulated *PIF4* mRNA expression compared with *elf3-1* in the dark (Figure 5A). In 12L/12D condition, the transcript level of *PIF5* was highest at ZT8 and decreased after ZT12 in WT (Figure 5C). The *cop1-4* mutant seedling showed down-regulated *PIF5* mRNA level compared with WT (Figure 5C). However, *elf3-1* mutant seemed to up-regulated *PIF5* mRNA expression compared with Col-0 (Figure 5C). The *cop1-4elf3-1* double mutant seedling observed up-regulated *PIF5* mRNA expression level compared with *cop1-4* and down-regulated *PIF5* mRNA expression level compared with *elf3-1* (Figure 5C).

In SD condition, *PIF4* mRNA level was much higher in wild-type seedling than in mutants seedling at ZT8 (Figure 5B). And then, *PIF4* expression level slowly down after ZT12. *PIF4* mRNA level was much higher in *elf3-1* mutant than in WT and lower in *cop1-4* mutant than in WT in the dark (Figure 5B). The expression of *PIF4* in *cop1-4elf3-1* mutant was lower than in *elf3-1*, not followed that of *cop1-4* mutant in the dark (Figure 5B). During in the light (except ZT0), *PIF5* mRNA levels increased in all three mutants (Figure 5D). After ZT12, the *cop1-4* mutant seedling slightly showed down-regulated *PIF5* mRNA expression compared with Col-0 (Figure 5D). However, the expression level of *PIF5* is significantly elevated in *elf3-1* mutant compared to WT (Figure

5D). The *cop1-4elf3-1* double mutant seedling displayed up-regulated *PIF5* mRNA expression level compared with *cop1-4* mutant and down-regulated *PIF5* mRNA expression level compared with *elf3-1* mutant (Figure 5D).

We found that COP1 acts downstream of EC complex in genetic analysis of hypocotyl length (Figure 1). Therefore, the transcription levels of *PIF4* and *PIF5* were partially suppressed in *cop1-4elf3-1* double mutant, not following those of cop1-4. These data suggest that increased expression levels of *PIF4* and *PIF5* in *cop1-4elf3-1* compared with *cop1-4* contribute to hypocotyl elongation of photomorphogenesis in *PIF*-indendent pathway. In previous study, COP1 protein function acts as repressor of photomorphogenesis and has many roles such as mediating the regulators, *HY5* or bZIP transcription factor. Taken together, these data suggest that *PIFs* synergistically repress photomorphogenesis as interacting with COP1 and EC complex proteins.

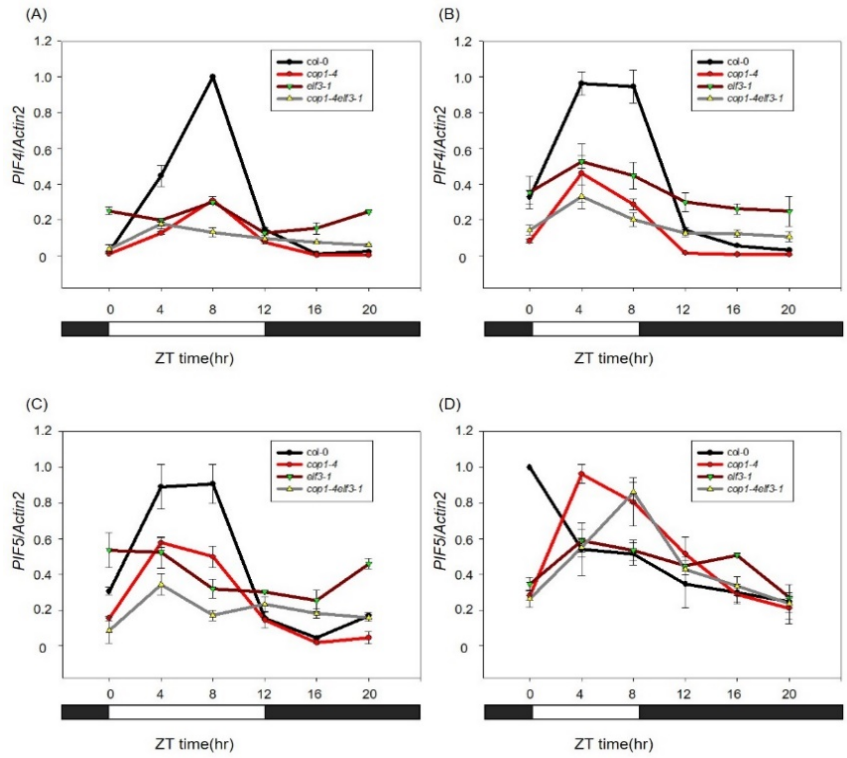

Figure 5. The *PIF4* and *PIF5* levels are partially suppressed in *cop1-4elf3-1*.

(A-B) *PIF4* and *PIF5* mRNA expression patterns in *cop1-4*, *elf3-1*, *cop1-4elf3-1* and wild type (Col-0). Total RNA was isolated from 12-d-old in 12h light/12h dark grown seedling for qRT-PCR assays (n ≥ 20). *ACTIN2* was used as an internal control. Error bars indicate standard deviation. Seeds were grown on MS medium with 2% sucrose.

(C-D) *PIF4* and *PIF5* mRNA expression patterns in *cop1-4*, *elf3-1*, *cop1-4elf3-1* and wild type (Col-0). Total RNA was isolated from 5-d-old in 8h light/16h dark grown seedlings for qRT-PCR assays (n ≥ 20). *ACTIN2* was used as an internal control. Error bars indicate standard deviation. Seeds were grown on MS medium with 2% sucrose.

EVENING COMPLEX mediates *COP1* transcriptional regulation

To further investigate the functional relevance of the observed *cop1-4* phenotype (Figures 1 to 2), we compared *COP1* mRNA expression in wild-type seedling with that of *elf3-1*, *elf4-101* and *pcl1-1* mutant seedlings (Figure 6). To demonstrate the mechanism for suppression of photomorphogenesis by EC complex, we analyzed transcript level of *COP1* under LD conditions or SD condition (Figure 6). All samples were collected every 4 hours after light-on (ZT0) from seedling in 12d-old or 5d-old, respectively.

In 12L/12D condition, the transcript level of *COP1* was highest at ZT8 and decreased after ZT8 in Col-0 (Figure 6A). In previous study, ELF3 protein accumulation approaches a maximum level just before the onset of the darkness [12, 47]. Under 12L/12D condition, the *elf3-1*, *elf4-101* and *pcl1-1* mutants slightly seem to up-regulated *COP1* mRNA expression level compared with Col-0 at ZT12 (Figure 6A). Similarly, under SD condition, the transcript level of *COP1* was slightly increased compared with Col-0 at ZT8 (Figure 6B).

The transcript level of *COP1* showed no significant difference in *elf3-1*, *elf4-101* and *pcl1-1* mutants under 12L/12D (at ZT12) and SD (at ZT8) condition. *COP1* transcript level was slightly up-regulated in evening complex mutants which differentiate to WT. These results prove that *EC* complex functions at the upstream of COP1 and represses *COP1* transcriptional level. However,

the double (*cop1-4elf3-1*, *cop1-4elf4-101*, *cop1-4pcl1-1*) and triple (*cop1-4elf3-1elf4-101*) *cop1-4* background mutants strikingly showed severe phenotype following *cop1-4* single phenotype (Figures 1 to 2). Thus, we suggest that EC complex is related to translational regulation of COP1. Since there was no difference in transcriptional level (Figure 6), EC complex and COP1 were thought to have protein interaction with control in translational regulation. We can imagine strong translational regulation between EC complex and COP1.

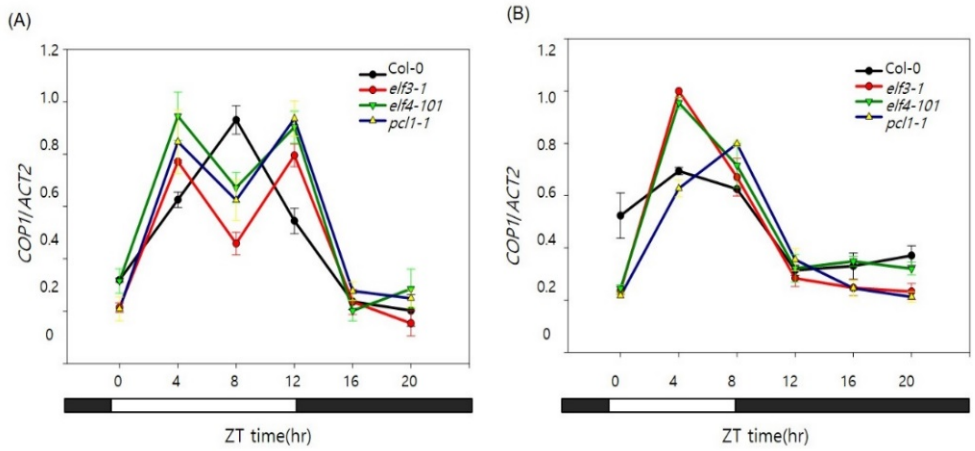

Figure 6. *COP1* mRNA level slightly increases in *elf3-1*, *elf4-101* and *pcl1-1* mutants.

Expression level of photomorphogenesis-related *COP1* in *elf3-1*, *elf4-101* and *pcl1-1*. qRT-PCR were performed with total RNA from seedlings at (A) 12d-old seedling under 12L/12D condition and (B) 5d-old seedling under SD condition, respectively. Samples were collected 4 hours after light-on (ZT0). The data were obtained from three independent biological replicates.

COP1 physically interacts with ELF3, ELF4 and LUX by *in vitro* Yeast two hybrid assays

According to previous study, it was reported that COP1 and ELF3 function toward GI destabilization played an important role in the regulation of light signaling to the clock and the control of the expression modes of flowering time genes [48]. Based on this study, we hypothesize that ELF3 has a relationship with COP1 in regulating *PIFs* mRNA level (Figures 3A to 3D). *COP1* mRNA expressison slightly was increased in evening complex mutants, however, there was almost no difference (Figures 6A to 6B). So we suggest that between EC complex and COP1 regulates translational level process. To examine whether COP1 interacts with ELF3, ELF4 and LUX (EVENING COMPLEX), we performed yeast two hybrid assays. The full length of COP1 (aa;1-2028) was used as bait in pGBK vector. For preys, it was used the full length of ELF3 (aa;1-2088), ELF4 (aa;1-336), and LUX (aa;1-975). COP1 baits in pGBKT7 vectors and ELF3, ELF4 and LUX preys in pGADT7 vectors were cotransformed into yeast strain AH109 (Figure 7A). Then, we conducted the selection of the transformants on minimal media lacking adenine, leucine, histidine and tryptophan.

In previous study, we know that full-length COP1 interacts with ELF3 as a positive control [48]. Therefore, we measured β-galactosidase activity, which expressed two proteins interaction through CPRG. These revealed that COP1

interacted with ELF3 as the same results in previous study. COP1 interacted with ELF4, slightly 0.41 times than negative control. Also, COP1 interacted with LUX, slightly 0.21 times more than negative control. Taken together, these results indicate that COP1 very weakly interacts with ELF3, ELF4 and LUX, independently.

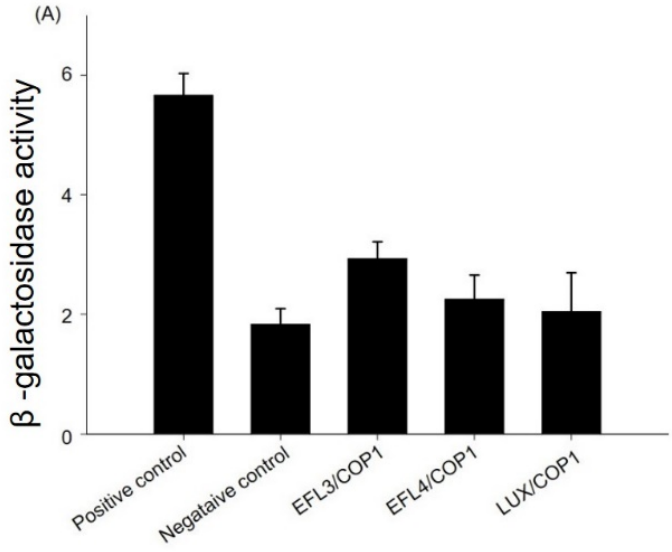

Figure 7. *In vitro* COP1 interacts with EVENING COMPLEX in yeast two hybrid assays.

(A) COP1 interacts with ELF3/ELF4/LUX, in β-galactosidase activity, respectively. COP1 was used as bait in pGBK vector. ELF3, ELF4 and LUX were used as preys in pGAD vector. The following preys, cloned into pGAD vector, were used: pGADT7 (AD) and pGBKT7 (BD), as a positive control, empty pGADT7 (AD) and pGBKT7 (BD), as a negative control. SD medium (-LWHM; lacking medium of tryptophan, leucine, histidine, and adenine) was used for selection of the interaction between bait and prey proteins. Means and standard deviations were obtained from three biological replicates. These experiments were repeated three with the same results.

DISCUSSION

COP1 is involved in repression of photomorphogenesis in the ubiquitination pathway, which degrades several transcription factors such as HFR1, HY5 and CO [38, 49-52]. ELF3-ELF4-LUX (called evening complex) complex functions as the molecular basis for circadian gating of hypocotyl growth in the early evening, regulating of *PIF4* and *PIF5* mRNA expressions [12]. ELF3 gene as a protein oscillation in various photoperiodic conditions is associated with circadian clock function. [13, 47] [53]. However, the function of between ELF4, LUX and *PIFs* in the regulation of hypocotyl elongation remains elusive through *COP1* in photomorphogenesis. In this study, we provide several pieces of evidence showing how EC complex regulates the suppression of *PIFs* expression through COP1. COP1 has a relationship with EC complex. To determine this, we examined the phenotype of double and triple mutants in 12L/12D, LD, SD and DD conditions (Figures 1 to 2). We compared the phenotype of *cop1-4* background mutant lines (double and triple mutants) with wild type (Col-0). We obtained the result indicating that *cop1-4* mutation had an effect on the photomorphogenesis for regulating hypocotyl elongation. Because of affecting the expression pattern of hypocotyl elongation-responsive genes such as *PIFs*, the *cop1-4* crossed mutants observed short hypocotyl length the same as the *cop1-4* single mutant. The reduced

transcriptional repression levels of *PIF4* and *PIF5* are regulated by EC complex [12, 17]. Taken together, the phenotype observation of single, double, and triple mutants showed *cop1-4* mutant phenotype (Figures 1 to 2). Therefore, COP1 is epistatic to EC complex. In previous study, ELF4-HA can co-immunoprecipitate both ELF3 and LUX, however LUX did not co-immunoprecipitate with ELF4-HA. These results observe that ELF3 is a crucial for *in vivo* formation of the EC complex. Furthermore, hypocotyl length *elf3-1elf4-3* and *elf3-1lux-4* double mutants grown under a 12L/12D condition did not show additive effects over *elf3* [12]. According to this results, we imagine that ELF3, ELF4 and LUX function together as a complex to regulate common pathways [12, 17]. In this study, we already showed that between crossed *cop1-4* background mutants (Figures 1 and 2). As a further study, we recommend that *cop1-4elf3-1elf4-101pcl1-1* quadruple mutant phenotype should be examined. These further experiment of the genetic analysis will provide more insights into the mechanisms of hypocotyl elongation, following *cop1-4* mutant phenotype.

To investigate how EC complex is regulated by *PIFs* through COP1, we next analyzed that the transcript levels of *PIF1*, *PIF3*, *PIF4* and *PIF5* in *cop1-4* were down-regulated compared with Col-0 (Figure 3A). *PIF* direct-target genes also down-regulated in *cop1-4* compared with Col-0 (Figure 4A). These results indicate that the induction of hypocotyl growth is caused in part through *PIFs*. In other words, COP1 activates the transcription of *PIFs*

expression level (Figures 3 to 4). A previous study revealed that EC complex repressed *PIF4* and *PIF5* mRNA levels. Thus, we were focused to study these genes in various mutant lines, especially *cop1-4elf3-1* double mutant (Figures 5A to 5D). In photomorphogenesis, *PIF4* and *PIF5* mRNA levels in *cop1-4elf3-1* double mutant were lower than those of in *elf3-1* single mutant. Therefore, it is possible that *COP1* may partially affect *PIFs* expression levels by regulating of hypocotyl length in phoromorphogenesis. In addition, we found that *COP1* mRNA level slightly increased in *elf3-1*, *elf4-101* and *pcl1-1* mutants (Figures 6A to 6B). However, there was no significant difference in *COP1* expression in evening complex mutants, compared to wild type. Therefore, we imagine that this results are related by protein interaction to control photomorphogenesis in translational regulation. Genetic analysis show that *COP1* acts with *EC* complex in photomorphogenic pathway, indicating that *COP1* is epistatic to EC complex (Figures 1 to 2). We subsequently examined physical interaction between COP1 and EC complex by yeast two hybrid assay. Since there was no difference transcript level of *COP1,* we described a regulatory mechanism that COP1 may interact with EC complex (Figure 7). Based on yeast two hybrid assay (Figure 7), we strongly get results, indicating translational data. Previous study show that COP1 interacts with ELF3 by interacting proteins. Also, ELF3 has an important bridge role which is necessary to interaction between ELF4 and LUX [12]. Because ELF3 is a crucial gene to bridge, the results of weak

protein interactions between COP1 and EC complex was observed (Figure 7). For *in vivo* interaction, further analysis of bimolecular fluorescence complementation (BiFC) assays of the interactions between COP1 and EC complex in the nucleus of onion epidermal cell is required. Using transient expression assays in onion epidermal cells, we will find that between COP1 physically interacts with ELF3, ELF4 and LUX *in vivo*. Also, further analyze is necessary to determine whether COP1 and ELF4 and LUX co-immunoprecipitate from transgenic plants expressing tagged proteins *in vivo*. These further analysis of the protein interaction between COP1 and EC complex will provide more insights into the mechanisms of photomorphogenesis through *PIFs*. In conclusion, we propose a new function between EC complex and *PIFs* as a repressor in the photomorphogenesis through COP1 in this study.

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국 문 초 록

식물의 생체시계 (circadian clock)란 식물이 하루24시간 주기에 맞춰 살아가며 그들의 성장과 발달을 조절하는 일련의 생화학적 기작을 뜻한다. 이러한 생체시계 유전자들은 계절적, 환경적 변화에 반응하며 식물체 내부에 존재하는 생체시계의 신호 전달에 따라 식물의 세포분열, 신장조절에 영향을 미친다. 생체리듬 조절 유전자로 알려진 EVENING COMPLEX (ELF3-ELF4-LUX)는 이른 저녁 시간에 형성되는 단백질 복합체이며 *PIF mRNA*의 발현량을 억제함으로써 hypocotyl 길이를 조절한다고 알려져 있다. COP1 (CONSTITUTIVE PHOTOMORPHOGENIC1) 단백질은 애기장대의 암 조건에서 광형태형성 (photomorphogenesis)을 억제하는 능력을 가진 단백질이며 유비퀴틴 기작에서 타켓이 되는 단백질을 분해 (degradation)한다고 알려져 있다. *EVENING COMPLEX- EARLY FLOWERING4* (*ELF4*)-*EARLY FLOWERING3* (*ELF3*)-*LUX ARRHYTHMO* (*LUX* ; also known as *PHYTOCLOCK1*)가 광형태발생억제자 (repressor of photomorphogenesis)로 알려진 *COP1*을 통해 hypocotyl 길이 조절에 관여하고 있음을 밝히기 위해 표현형을 살펴보았다. 따라서 *COP1* 돌연변이체인 *cop1-4*을 evening complex 돌연변이체와의 교배를 통해 유전학적 분석을 실시하였다. 그 결과 *cop1-4* 돌연변이체는 hypocotyl 길이가 Wild type보다 짧은 표현형을 보이고, 각각의 *EVENING COMPLEX*의 돌연변이체 *elf3-1*, *elf4-101*, *pcl1-1*은 hypocotyl 길이가 Wild type

보다 긴 표현형을 보였다. 각각의 *cop1-4elf3-1*, *cop1-4elf4-101*, *cop1-4pcl1-1* double mutant와 *cop1-4elf3-1elf4-101* triple mutant가 *cop1-4*과 같은 짧은 hypocotyl 표현형을 보였다. 즉, *ELF3*, *ELF4*, *LUX*는 *COP1*을 통해 hypocotyl 길이를 억제한다는 것을 밝혔다. ELF3, ELF4, LUX가 *PIF4, PIF5* mRNA발현량을 억제한다고 알려져 있기 때문에 COP1이 hypocotyl 길이를 억제하기 *PIFs* mRNA 발현량을 조절하는지 살펴보았다. 그 결과, *cop1-4* 돌연변이체에서 *PIF1*, *PIF3*, *PIF4*, *PIF5*의 *mRNA*발현량이 감소하는 현상을 보였으며 이는 COP1이 ELF3, ELF4, LUX처럼 *PIFs*의 전사인자를 조절한다는 사실을 밝혔다. *EVENING COMPLEX*와 *COP1*과의 관계를 정확히 분석하기 위해 분자유전학적 분석을 하였다. 12h light/12h dark (or 8h light/16h dark) 조건에서 *elf3-1*, *elf4-101*, *pcl1-1* 돌연변이체에서 *COP1*의 mRNA발현량을 살펴보았다. 그 결과, 밤시간에 *COP1* mRNA의 발현에 차이가 거의 없었다. ELF3, ELF4, LUX 단백질이 *COP1* mRNA 발현에 영향을 주지 않으며 이들이 모두 *PIFs* pathway를 통해 hypocotyl 길이를 조절한다는 것을 확인하였다. COP1 전사조절에 영향을 주지 않았기 때문에 이들의 단백질 관계를 알아보기 위해 yeast two hybrid를 실시하였다. 그 결과 ELF3, ELF4, LUX 단백질이 COP1 단백질과 상호결합을 하고 있음을 확인하였다. 이로써, 광형태형성 경로에서 ELF4-ELF3-LUX 단백질은 COP1 단백질의 기능을 조절하여 hypocotyl의 길이를 억제하는 역할을 한다는 가능성을 제시하고 있다.